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13. ABSTRACT ***(Maximum 200 Words)***

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis.

The proposal is based upon our previous experiments, primarily performed in conventional cell culture models, demonstrating that maspin has tumor suppressor activity. Recently, we have established transgenic mice overexpressing maspin in the mammary gland, and generated maspin knockout mice. We have crossed maspin transgenic mice with a breast tumor WAP-Tag strain. Our data suggest that maspin functions directly as an inhibitor for angiogenisis and metastasis. We have also shown in this report the mechanism by which maspin inhibits normal mammary development and reduced tumor progression in bigenic mice. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

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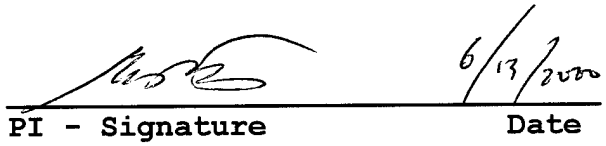

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Table of Contents

Introduction

Breast cancer is the most common malignancy in western countries. Among women in the United States between the ages of 40 and 55 years, breast cancer is the leading cause of death (1). In the past, study of breast cancer has lagged behind other forms of cancer. Although the balance is being shifted, principally owing to women's awareness and increased funding, there are great gaps in our understanding of almost every aspect of this disease. Recently, The National Cancer Institute established a Breast Cancer Progress Review Group (BCPRG) to identify and prioritize scientific needs and opportunities that are critical to hasten progress against the disease. The proposal by this group has provided a guideline for individual breast cancer researcher to follow (Web site for this proposal: http://wwwosp.nci.nih.gov/planning/prg).

The object of this proposal is to understand the tumor suppressor function of maspin, a novel serine protease inhibitor, and to test directly maspin as a therapeutic agent for breast cancer. Transgenic and knockout mouse models will be employed to study the effects of gain and loss of maspin function on mouse mammary tumorigenesis and development. We hypothesize that overexpression of maspin should be protective against mammary tumorigenesis and metastasis, while loss of maspin will render mice more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing these mice with other well characterized mouse breast cancer models to test the tumor suppressor activity of maspin. Mammary tumorigenesis and normal mammary development will be studied using a variety of established techniques, including histopathology and whole mount analyses. Finally, we plan to deliver maspin locally as a drug to determine its therapeutic efficacy as an anti-tumor agent.

The following tasks were proposed for the first 12 month period of study.

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice. Months 1-24. Total 100 mice will be used.

a. Generate four groups of mice by crossing WAP-maspin heterozygotes with WAP-Tag heterozygotes.
b. Continuous mating to activate the transgenes.
c. Collect mammary gland samples for histopathology and other studies.
d. Record tumor progression, and take tumor samples for histopathology.

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20. Numbers of mice: 40

a. Prepare large quantity of recombinant GST-maspin protein and make maspin Evac pellets.
b. Implant pellets in the mammary fat pad near the site of mammary tumor development in WAP-Tag mice.
c. Biopsy of tumor samples, examine proliferation and apoptosis.

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression. Months 6-24. Number of mice: 40

a. Do proliferation and apoptosis experiments using mammary gland biopsies from WAP-maspin mice to study effects on lobuloalveolar development.
b. Deliver maspin pellets to the mammary gland of normal 4-6 week old virgin mice and post-lactation mice to study effects on ductal morphogenesis and involution.

Task 5. Effect of maspin gene disruption on mammary tumorigenesis and development. Months 12-36. Number of mice: 200.

a. Gene knockout mice , if viable analyze mammary gland development.
b. Induce mammary tumorigenesis by DMBA in KO and control mice, observe tumor progression and metastasis.

Body

Materials and methods

Animals

WAP-Tag mice were provided by Dr. Priscilla Furth as a collaboration. WAP-maspin transgenic mice were established in this laboratory. Maspin heterozygous mice were generated in this laboratory. Mouse tumors were provided by Dr. Dan Medina. All animals were maintained within the PI's animal facility at Baylor.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. Anti-Tag antibody was purchased from Pharmingen. All secondary antibodies were purchased from Zymed, Inc.

Northern and Western analysis

RNAs and proteins were isolated from mammary glands from virgin, pregnant, lactating stages. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 ug RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Mammary glands were removed under anesthesia from normal and transgenic females at different stages of development. Mammary tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin (AbS4A) (2). The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The

sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For PCNA staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit. The anti-Tag antibody (pharminogen) will be diluted 1:400 times and used as instructed by Pharmingen.

Results and Discussion

Task 1. Analysis of the tumor suppressor activity of maspin by crossing WAP-maspin mice with WAP-Tag mice.

WAP-Tag transgenic mice (3) that are highly susceptible to mammary tumorigenesis were mated with WAP-maspin to generate four groups of mice: Tag +/maspin+ (25), Tag+/maspin-(15), Tag-/maspin+(16), Tag-/maspin-(15). They were mated continously to activate transgene expression. Tumor progression data were recorded. Tumors first appeared in Tag+ mice about three months after first pregnancy, with 100% of them developed tumors by 6 months. Tumor growth was monitored by measurement with caliper biweekly and was followed until the tumor size reached 2.0 cm in diameter or tumor volume reached 10 % of body weight before the mice were sacrificed. At the moment, all mice were sacrificed and their samples harvested. Tumor free curves between biogenic and Tag+ were compared. No significant difference was observed for tumor incidence between these two groups (Fig. 1). We conclude maspin overexpression does not prevent tumor initiation. Lung tissues were collected from tumor mice. Tumor metastasis to lung was evaluated by examining lung sections by H&E staining. Our data showed tumor metastasis were reduced in bitransgenic mice (37.5%) comparing to the Tag+ mice (56.7%). It will be further examined by immunohistochemical analysis using an anti-Tag antibody. Current data indicate that overexpression of maspin in vivo can block tumor metastasis.

The mechanism of metastasis inhibition in biogenic mice was investigated. Tumor sections were analyzed for apoptosis rate and microvessel density by TUNEL assay and CD31 immunostaining.
Preliminary data show that tumors from bigenic samples have increased apoptosis and decreased MVD, suggesting maspin may inhihit tumor progression by its anti-angiogenic property.

To further delineate maspin's role in angiogenesis inhibition, we carried out both endothelial cell motility assay and in vivo rat corneal assay. We demonstrated that maspin was an effective inhibitor of angiogenesis. In vitro it acted directly on cultured endothelial cells to stop their migration towards bFGF and VEGF and to limit mitogenesis and tube formation. In vivo it blocked neovascularization in the rat cornea pocket model. Maspin derivatives mutated in the serpin reactive site lost their ability to inhibit the migration of fibroblasts, keratinocytes and breast cancer cells but were still able to block angiogenesis in vitro and in vivo. When maspin was delivered locally to human prostate tumor cells in a xenograft mouse model, it blocked tumor growth and dramatically reduced the density of tumor-associated microvessels (4).

Task 3. Tumor inhibition by local Evac implants of maspin. Months 6-20.

We have prepared maspin Evac to delivery maspin to mammary tumors. Instead of using WAP-Tag tumor mice, we started by using Balb/c mice and tumor tissues isolated from syngenic Balb/c mice since this model was well characterized by Dr. Dan Medina (5). All tumors and mice were provided by Dr. Daniel Medina as a collaboration. The study was carried out as followings. Tumor tissues were microscopically dissected into pieces for implanting to the fat pad of 8 week old Balb/c female mice. They were allowed to grow for two weeks inside mammary gland. Maspin slow release Evacs were surgically implanted beside the tumor sites and the incisions were closed. Two weeks later, tumors were excised and their sizes were measured. Initial experiment showed maspin treated tumors have reduced size but due to the large variation of tumor size and small scale of first experiment, the difference was not proved to be statistically significant. In view of recent data from biogenic experiment, it is also possible that maspin delivered may not be sufficient to inhibit tumor growth. Another possibility is that the delivery efficiency needs to be optimized. We are currently developing lipsome-maspin mediated gene delivery. The data will be collected in the next 12 months.

Task 4. Characterization of physiological functions of maspin by mean of targeted overexpression. Months 6-24.

We have found that overexpression of maspin at midpregnancy inhibits mouse mammary gland development (6). One hypothesis is that maspin may be involved not only in extracellular matrix remodeling but also as a regulator for cell proliferation. On the other hand, over-expression of maspin may inhibit mammary gland development by inducing extensive apoptosis. To understand whether the induction of transgene expression caused any changes of alveolar cells in proliferation and apoptosis, PCNA staining and TUNEL assay were carried out with midpregnant mammary samples. Our data indicate that the maspin transgene does not change the proliferation rate, but increases significantly the apoptosis of alveolar cells. The increase was sustained even to the late stage of pregnancy when normal gland had little apoptosis. Since milk protein genes can function as differentiation markers for the mammary gland, we compared their expression patterns in transgenic and wildtype control mice. Western blot analysis showed that WAP and β-casein were highly expressed in wild-type mammary gland at day 19 pregnancy and throughout the three stages of lactation. However, neither WAP nor β-casein was detected in our assay at day 19 pregnancy in transgenic glands as compared to control. The levels of both milk proteins were present in lactating day 1 transgenic glands, but at a reduced level. Following three days of lactation, WAP and β-casein levels in the transgenic mice increased to that of control. The decreased expression of milk genes could arise from the reduced number of alveoli, as well as lower expression by each alveolar epithelial cell.

In summary, maspin transgene expression resulted in a decrease in both the number of lobular-alveolar structures and the size of each alveolar unit during pregnancy and early lactation, and this effect is due to the increased rate of apoptosis.

Task 5. Effect of maspin gene disruption on mammary tumorigenesis and development.

We have generated maspin knockout mice in order to examine maspin's role in mammary tumor progression. Unfortunately, the homozygotes are lethal in embryo development. That limited our ability to study the mammary development at late stages. The heterozygotes were viable but we did not observe any defect in mammary gland development by histological analysis. However, we decided to examine whether the heterozygotes might display some phenotypes when they were

challenged by chemical carcinogen DMBA. The experiment is currently ongoing and no data are available at the moment. Since this set of experiment is nearly a duplicate of original task 2, we decide to cancel task 2 and focus on the effect of loss of maspin on DMBA-induced tumorigenesis.

Key research accomplishments

- Overexpression of maspin in transgenic mice inhibits mammary gland development.
- Targeted overexpression of maspin reduced the rate of tumor progression and metastasis.
- Maspin is a potent angiogenesis inhibitor.
- Maspin knockout mice are homozygous lethal. Heterozygous mice may display phenotypes after being challenged by chemical carcinogen.

Reportable Outcomes

Two papers were published with the support of this grant proposal. One manuscript on bigenic mice study (task 1) is in preparation. Please see attached papers in appendix for the publication record.

1. Zhang, M., Magit, D., Botteri, F., Shi, Y., He, K., Li, M., Furth, P., Sager, R. Li, M., Furth, P., Sager, R. Maspin plays an important role in mammary gland development. **Developmental Biology**, 215: 278-287, 1999.

2. Zhang, M., Volpert, O., Shi, Y., Bouck, N. Maspin is an angiogenesis inhibitor. **Nature Medicine**, 6, 196-199, 2000.

3. Zhang, M., Shi, Y., He, K., Magit, D., Furth, P., Pardee, A., and Sager, R. Reduced breast tumor metastasis in mice overexpressing maspin. In preparation.

Conclusion

All four tasks proposed in the grant were initiated or carried out in the first two years of proposal. We have obtained very informative data suggesting maspin functions directly as a metstasis inhibitor. We have uncovered the mechanism by which maspin inhibits normal mammary development during pregnancy in WAP-maspin transgenic mice, and we are testing maspin against tumor progression in mice. We have also demonstrated that maspin inhibits angiogensis. Continuation of these tasks in the next few years will help us understand the role of maspin in tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of breast cancer.

Reference

1. American Cancer Society. Cancer statistics. CA 1992; 42:30-31.

2. Zou, Z., Anisowic, A., Hendrix, M.J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. Identification of a novel serpin with tumor suppressing activity in human mammary epithelial cells. Science, 263:526-529, 1994.

3. Li, M., Hu, J., Heermeier, K., Hennighausen, L. & Furth, P.A. Apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways. *Cell Growth Differ* **7**, 13-20 (1996).

4. Zhang, M., Volpert, O., Shi, Y., Bouck, N. Maspin is an angiogenesis inhibitor. **Nature Medicine**, 6, 196-199, 2000.

5. Kittrell, F.S., Oborn, C.J. & Medina, D. Development of mammary preneoplasias in vivo from mouse mammary epithelial cell lines in vitro. *Cancer Res* **52**, 1924-1932 (1992).

6. Zhang, M., Magit, D., Botteri, F., Shi, Y., He, K., Li, M., Furth, P., Sager, R. Li, M., Furth, P., Sager, R. Maspin plays an important role in mammary gland development. **Developmental Biology**, 215: 278-287, 1999.

Appendices

See attached (one original and 2 copies).

Maspin is an angiogenesis inhibitor

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Maspin, a unique member of the serpin family, is a secreted protein encoded by a class II tumor suppressor gene whose downregulation is associated with the development of breast and prostate cancers[1,2]. Overexpression of maspin in breast tumor cells limits their growth and metastases *in vivo*. In this report we demonstrate that maspin is an effective inhibitor of angiogenesis. *In vitro*, it acted directly on cultured endothelial cells to stop their migration towards basic fibroblast growth factor and vascular endothelial growth factor and to limit mitogenesis and tube formation. *In vivo*, it blocked neovascularization in the rat cornea pocket model. Maspin derivatives mutated in the serpin reactive site lost their ability to inhibit the migration of fibroblasts, keratinocytes, and breast cancer cells but were still able to block angiogenesis *in vitro* and *in vivo*. When maspin was delivered locally to human prostate tumor cells in a xenograft mouse model, it blocked tumor growth and dramatically reduced the density of tumor-associated microvessels. These data suggest that the tumor suppressor activity of maspin may depend in large part on its ability to inhibit angiogenesis and raise the possibility that maspin and similar serpins may be excellent leads for the development of drugs that modulate angiogenesis.

To study the potential anti-angiogenic properties of maspin, the mouse protein was produced in *Escherichia coli* as a recombinant glutathione S-transferase (GST) fusion protein and tested in a variety of angiogenesis assays. Recombinant maspin blocked endothelial cell migration induced by vascular endothelial growth factor (VEGF) in a dose dependent manner with a median effective dose (ED_{50}) of 0.2 μM–0.3 μM (Fig. 1*a*). Similar results were obtained using basic fibroblast growth factor (bFGF) as an inducer (Fig. 1*b*; data not shown). At 1 μM, maspin completely blocked the response of the endothelial cells to both angiogenic inducers, whereas the GST control was inactive. Maspin also inhibited the growth of endothelial cells (Fig. 1*c*) and prevented them from forming tubes in a matrigel assay (data not shown).

Purified maspin effectively inhibited neovascularization *in vivo*. Rat corneas were surgically implanted with non-inflammatory, slow release pellets containing maspin with bFGF and examined 6 or 7 days later for ingrowth of vessels. Maspin completely blocked bFGF-induced neovascularization (Fig. 2; compare i and v).

Maspin, a member of the large family of serine protease inhibitors (serpins), has been shown to serve as a substrate rather than an inhibitor for trypsin-like serine proteinases[3], suggesting that it may fall into the growing category of noninhibitory serpins that lack antiprotease activity. One recent study, using purified reagents *in vitro*, suggested that maspin might have some antiprotease activity as it inhibited tissue plasminogen activator in vitro[4]. We were unable to confirm these results in our laboratory[5], but to determine if the anti-angiogenic activity of maspin depended on the inhibition of some undefined protease, we constructed, expressed, and tested several mutants. The RSL (reactive serpin loop) near the *C*-terminus of serpin family members is essential for their antiprotease activity. Mutations at the RSL region of other serpins, especially at the P1 site, abolish serpin activity[6]. To disrupt this loop in maspin two different mutations were introduced in the RSL region: a *C*-terminal deletion downstream of P7′ residue[7] (maspin ΔRSL) and a conversion of the critical P1 arginine of the RSL loop to a glutamine (maspin*). A third mutant was constructed in which the first 139 amino acids were removed but the serpin region was left intact (maspinΔN). We tested recombinant maspin and two RSL mutants in a quantitative tissue-type plasminogen activator (tPA) assay. As previously demonstrated[5], neither wild-type maspin nor its derivatives displayed any tPA inhibition (data not shown).

We used migration assays, with nonendothelial cells, to demonstrate that the constructed mutants were indeed defective. An intact RSL region is essential for maspin to block the migration of breast tumor cells *in vitro*[8]. When we repeated this assay with mutant proteins both maspin* and maspinΔN had no inhibitory effect (Fig. 1*d*). All three mutants were also unable to inhibit the migration towards bFGF of normal human fibroblasts (Fig. 1*e*) or normal human keratinocytes (Fig. 1*f*) although wild-type maspin protein produced in the same way was an effective inhibitor.

When we tested these defective mutants on endothelial cells (see Fig. 1*b* and *c*), they behaved somewhat differently. Those with RSL defects that were unable to block the migration of other cells, retained the ability to inhibit endothelial cell migration and mitogenesis. Protein with mutations in the RSL region also retained the ability to inhibit neovascularization *in vivo* (Fig. 2, compare i and vii). The N-terminal deletion, maspinΔN, was defective in all assays so it was not possible to determine if a crucial active region had been deleted or if it was just a dead protein.

To determine if the ability of maspin to inhibit angiogenesis is involved in its antitumor activity, we used an athymic mouse xenograft model. We implanted LNCaP prostate tumor cells subcutaneously on the bidorsal back of nude mice and monitored tumor growth and neovascularization after systemic treatment with exogenous maspin. Maspin-treated tumors contained considerably fewer vessels as determined by CD31 immunostaining than GST-treated controls (Fig. 3) To determine whether maspin effects on the tumor-induced vasculature were maintained during a more prolonged treatment, the above ex-

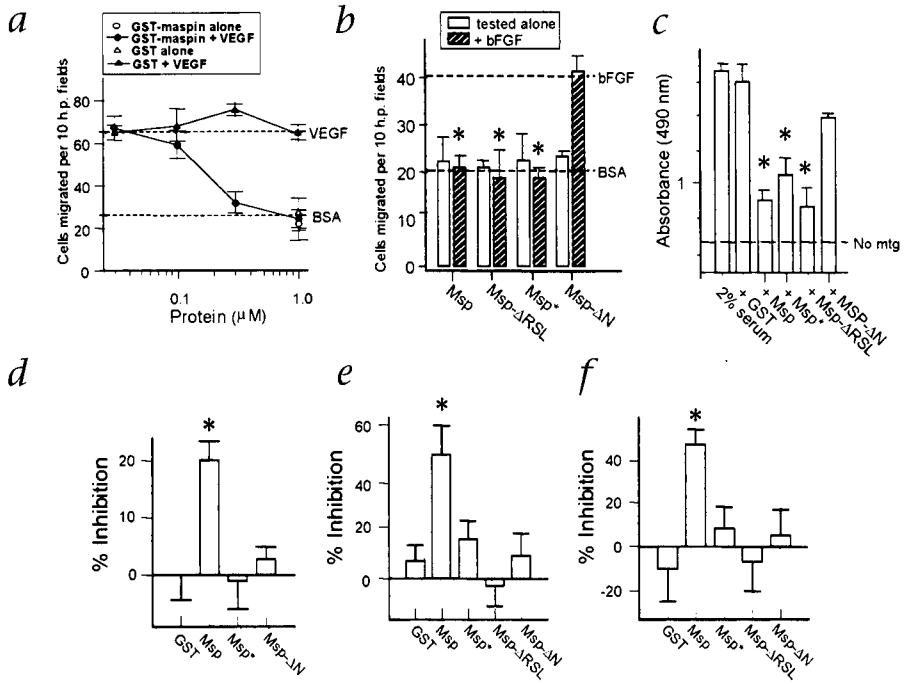

Fig. 1 Effect of maspin and its derivatives on cultured cells. ***a***, GST–maspin was tested at a range of concentrations for its ability to inhibit endothelial cell migration induced by 100 pg/ml VEGF. VEGF, migration towards VEGF alone; BSA, background migration in the absence of a gradient. ***b***, GST–maspin and its mutants (1 μM) were tested for their ability to inhibit endothelial cell migration towards 10 ng/ml bFGF. Maspin, GST–maspin fusion protein; maspin ΔRSL, maspin with a deletion at the C-terminus; maspin*, maspin containing an R to Q mutation in the P1 residue of the RSL loop; maspin ΔN, maspin with a deletion at the *N*-terminus. Glutathione-S-transferase tested alone was neutral in this assay. *, $P < 0.01$ compared with migration towards bFGF (bFGF-----). ***c***, Maspin and its mutants (1 μM) were tested for their ability to inhibit the growth of endothelial cells after 5 d. *, $P < 0.05$ compared with GST. ***d***, Maspin and its mutants were tested at a concentration of 0.3 μM for their ability to inhibit the invasion and migration of breast tumor cells. *, $P < 0.03$. ***e***, Maspin and its mutants were tested at a concentration of 1 μM for their ability to inhibit the migration of normal human fibroblasts towards bFGF (20 ng/ml). *, $P < 0.05$. ***f***, Maspin and its mutants were tested for their ability to block the migration of normal human keratinocytes towards 20 ng/ml bFGF. *, $P < 0.03$.

periment was replicated with tumors harvested after 7–8 weeks. We treated 32 tumor sites with maspin and 37 with GST. At week 8, the growth of 53% of the maspin-treated tumors had been completely inhibited. The remaining 15 maspin-treated tumors were reduced in size to 29.2% of GST control-treated tumors. The effect of maspin was reversible. Among those sites that exhibited no detectable tumors at week 8, most developed palpable tumors within 1–3 weeks after cessation of maspin treatment, indicating that some viable tumor cells remained.

To determine if the reduced size of maspin-treated tumors coincided with reduced neovascularization, we used 20 representative tumors from either maspin-treated (10 sites) or GST-treated tumors (10 sites) to quantify the density of microvessels after immunostaining with CD31 antibody (Fig. 3 and Table1). The density of vessels in maspin-treated tumors was reduced to 37.8% of control tumors and this difference was highly significant. We also compared the treated and control tumors of similar size. There was also reduction of vessel density (ranging from 33.3% to 45.5%, $n = 4$) in the maspin treated samples.

a

	Test substances	bFGF added	Corneal neovascularization +	+/-	-
i	**none**	+	6	0	0
ii	**GST**	-	0	0	3
iii	**GST**	+	3	0	0
iv	**Maspin**	-	0	1	6
v	**Maspin**	+	0	1	5
vi	**Maspin ΔRSL**	-	0	0	2
vii	**Maspin ΔRSL**	+	0	1	3
viii	**Maspin ΔN**	-	0	0	2
ix	**Maspin ΔN**	+	3	1	0

b

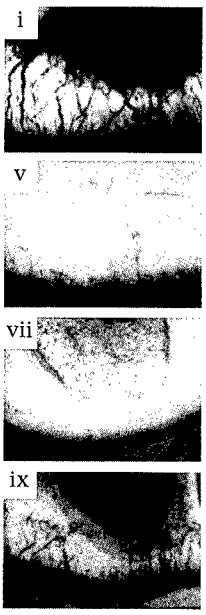

Fig. 2 Maspin inhibition of corneal neovascularization. Pellets containing 10 μM test substances at with or without 100 ng/ml bFGF were incorporated into Hydron slow-release pellets and implanted in rat corneas. After 6 or 7 d, rats were perfused with colloidal carbon to visualize vessels, and excised corneas were photographed with a ×20 objective (***b***) and scored for neovascularization (***a***). +/–, corneas in which only one or two vessels were induced that did not reach the pellet.

There is little evidence to indicate exactly how maspin blocks angiogenesis. It could act through a receptor-mediated event, as does thrombospondin-1[9]. The discrepancy in the effect of the RSL domain on endothelial and breast tumor cells may be due to the difference of receptors located on both cells. Alternatively, it could mimic the protease-independent effects of plasminogen activator inhibitor-1 (PAI-1). PAI-1 has a unique domain located at the *N*-terminal that regulates cell motility[10]. Experiments are underway to investigate this domain of maspin.

The observed ED_{50} of 0.2–0.3 μM in the capillary endothelial cell migration assay indicates that maspin is less potent than inhibitors like angiostatin (10 nM)[11], but more effective than small molecules such as captopril (10 μM)[12]. However, in epithelial tumor cells, exogenously added maspin localizes at the cell surface[13]. If this also happens on endothelial cells, then the concentration of soluble protein may not be particularly meaningful. Maspin was effective against several inducers *in vitro*, and *in vivo* it blocked angio-

genesis induced by bFGF and reduced tumor angiogenesis in response to the LNCaP, a cell line that produces VEGF as its major angiogenic factor (J. D. and N. B., unpublished data).

Complete inhibition of endothelial cell migration in vitro was achieved between 0.5 and 1 μM, in the same concentration range where maspin also inhibits tumor cell motility and invasion[13], but the mechanisms underlying these two maspin activities seem to be different. The former requires that the protein have an intact RSL (refs. 4, 8), whereas this feature was not essential for the inhibition of angiogenesis. Thus, even if maspin does inhibit some unidentified protease, this serpin activity is probably not involved in the inhibition of angiogenesis.

It is probable that maspin produced by tumor-associated normal tissue as well as that produced by developing tumor cells themselves can influence tumor growth. Maspin is produced selectively and at high levels by myoepithelial cells, which surround the normal mammary ducts. The myoepithelial cells themselves form only low-grade tumors and that they also may delay the progression of adjacent ductal carcinomas in situ to invasive carcinomas[14]. The anti-angiogenic nature of the maspin they secrete offers a possible explanation for both phenomena.

The ability to inhibit angiogenesis is only one of several activities associated with the intact maspin protein. Other serpins also have multiple functions and several of them are linked to angiogenesis and tumor growth. Plasminogen activator inhibitor-1 is involved in modulating both proteolysis and angiogenesis. Pigment epithelium-derived factor (PEDF), a known regulator of cell differentiation, is also a very potent anti-angiogenic factor[15]. Such results indicate that a variety of molecules, whose structure places them in the serpin family can be important regulators of natural tumor growth through their influence on neovascularization.

Table 1 Analysis of tumor volume and vessel number in mice treated with GST or GST–maspin

		GST	GST-maspin	*P*
Short term	Average of microvessel density[a]	28.6 ± 3.6 *n*=10	15.3 ± 1.8 *n*=10	0.001
	Total tumor sites	37	32	
	Percentage of complete inhibition[b]	2.7% (1/37)	53% (17/32)	0.0001
Long term	Average of tumor volume (mm^3) of sites with tumors	116.3 ± 26.1, *n*=36 (12.1–600)	33.9 ± 6.1, *n*=15 (9.0–87.5)	0.027
	Average of MVD	83.0 ±10, *n*=10 (38–143)	31.4 ±1.8, *n*=10 (21–42)	0.002

[a]Collected from three 'hot fields' (400×) in each tumor. [b]Complete inhibition indicates no sign of tumor or tumor size smaller than 2 mm in diameter after treatment. ±, standard error.

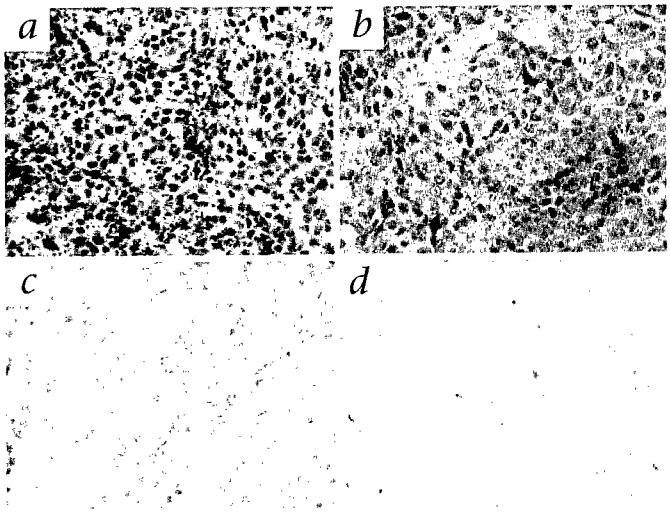

Fig. 3 Decreased tumor vessels after long-term treatment with exogenous maspin protein. Tumors were collected between 7 and 8 weeks from GST-treated mice (***a*** and ***c***) and from maspin-treated mice (***b*** and ***d***) and fixed and stained with hematoxylin and eosin (*a* and *b*) or with antibody against CD31 (*c* and *d*).

Methods

Cell culture. The human prostate carcinoma cell line LNCaP from American Type Culture Collection (ATCC; Manassas, Virginia) was grown in RPMI 1640 with 10% fetal bovine serum. MDA-MB-435 cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal calf serum. Normal human foreskin fibroblasts (HFF-S1) were established in the laboratory by S. Tolsma (Northwester College, Orange City, Iowa) and maintained in DME (Life Technologies) supplemented with 10% fetal calf serum. Normal human keratinocytes (NHOK) were a gift from M.Lingen (Loyola University Medical School, Maywood, Illinois) and maintained in keratinocyte growth medium (Clonetics Cell Systems, San Diego, California) with supplements recommended by the manufacturer. Bovine adrenal capillary endothelial cells, a gift from J. Folkman (Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115) were maintained in DMEM supplemented with 10% calf serum and 100 μg/ml endothelial cell mitogen, and were used at passage 15. Human dermal microvascular endothelial cells (HMVEC, passage 9) were from Clonetics Cell Systems and maintained in endothelial cell basal medium with 5% fetal bovine serum and an EC (endothelial cell) 'bullet' kit as recommended by supplier.

Protein production and purification. The pGST-maspin and maspinΔRSL vectors were constructed as described[7]. MaspinΔN was generated by digestion of the pGST-maspin vector with *Bam*H1, which was blunt ended and digested again with *Sst*1. The adhesive ends were filled by T4 polymerase and ligated to remove the N-terminal 139 amino acids. To construct pmaspin*, oligonucleotides encoding a mutation in the P1 residue were generated (sense primer 5′ –GGGTCCCAGATCTTA–3′ and antisense primer 5′– TAAGATCTGGGACCC–3′). Site-directed mutagenesis accomplished using the pGST-maspin vector and the above oligos (Stratagene, La Jolla, California). All mutant constructs were sequenced to ensure the in-frame fusion. The GST fusion proteins were produced as described[7]. The size and purity of recombinant maspin and mutants were confirmed by SDS-PAGE gel electrophoresis and western blot analysis using a polyclonal AbS4A antibody[7].

Endothelial cell assays. Migrations were done using bovine adrenal capillary endothelial cells as described[16]. Cells were starved overnight in DME containing 0.1% bovine serum albumin (BSA), collected, re-suspended in DME with 0.1% BSA, and plated at a concentration of 3×10^4 cells/well on the lower surface of a gelatinized 5.0 μm filter (Nucleopore, Pleasanton, California) in an inverted, modified Boyden chamber. Cells were allowed to adhere for 2 h at 37 °C, the chambers were re-inverted, test samples were added to the top wells and the chambers incubated 4 h at 37 °C to allow migration. Chambers were then disassembled, membranes fixed and stained and the number of cells that had migrated to the top of the filter in 10 high-power fields counted (a high power field is ×1000). DME supplemented with 0.1% BSA was used as a negative control to measure background resulting from random migration. All samples

were tested in quadruplicate.

To assess mitogenesis, HMVECs were plated at a concentration of 1×10^4 cells per well in gelatinized, 96-well microtiter plates, allowed to attach for 24 h and re-fed with endothelial cell basal medium (Life Technologies, New York) supplemented with 2% serum and GST or GST–maspin fusion protein and mutants at a concentration of 1 μM where indicated. The cells were incubated at 37 °C, with 5%CO_2 for 5 days to allow at least one population doubling. Mitogenesis was assessed using CellTiter nonradioactive proliferation assay (Promega) according to manufacturer's instructions. Baseline (no mitogen) reflects the value, determined before population doubling, 24 h after seeding.

Migration assays on non-endothelial cells. Breast tumor migration assays (MDA-MB-435) were done in quadruplicate as described[7]. After subtraction of background, data were normalized to give percent inhibition equating GST treated samples to 100% motility. For migration assays on fibroblasts and keratinocytes, HFF-S1 (0.7×10^6 cells/ml) and NHOK (1.2×10^6 cells/ml) were plated in serum-free basal media supplemented with 0.1%BSA on the bottom side of the microporous membrane (8-μm pore size) in the inverted modified Boyden chamber. The cells were allowed to attach for 1.5 h, the chambers were re-inverted and test substances in appropriate serum-free basal medium were added to the other side of the membrane. The migration was assessed, background was subtracted and data were reported as percent inhibition, as in tumor cell invasion assay, with bFGF induced motility normalized as 100%.

Corneal neovascularization assay. The assay was done as previously described[16]. Hydron pellets containing bFGF (100 ng/ml), GST (10 μM), GST-maspin (10 μM) or mutants (10 μM), alone in combination with bFGF were implanted into a pocket surgically created in avascular corneas of anesthetized female rats (Fisher 344: Harlan, Indianapolis, Indiana) 1–1.5 mm from the limbus. The compounds were used at concentrations at least 10-fold higher than in migration assay to account for the diffusion rate from a slow release pellet. Neovascularization was observed on day 6 or 7 after implantation. Vigorous growth of the blood vessels in the direction of the pellet was noted as a positive response. The animals were perfused with colloidal carbon, eyes were removed and fixed, corneas excised, flattened and photographed for a permanent record.

Tumor angiogenesis assay. LNCaP tumor cells were grown to 80% confluence, collected and resuspended in sterile HEPES-buffered salt solution at a concentration of 4×10^7cells/ml. This cell suspension was then mixed with Matrigel (Collaborative Research, Bedford Massachusetts) at a 1:3 ratio and added in 100-μl aliquotes into Eppendorf tubes containing maspin or GST, mixed on ice for 10 min and subsequently injected subcultaneously into the dorsal back of 5-week-old male athymic mice (K & K Universal, Freemont, California). Each mouse was inoculated at two to four sites. Ten mice were used for the initial vessel formation assay and 20 mice, for the tumor inhibition study. Primary tumors at the site of the subcutaneous injection were measured using calipers, and tumor volume was calculated according to the algorithm: length x $[\text{width}]^2 \times 0.5$ (ref. 17).

Ethylene/vinyl acetate copolymer (Evac; NEN) slow release pellets, containing maspin and GST, were prepared as described[18,19]. A lyophilized mixture of BSA and varied amounts of recombinant proteins was dispersed in 0.125 ml Evac dissolved in dichloromethane (Sigma). This mixture was frozen, dried, and cut into pellets of appropriate size. Each Evac pellet contained about 210 μg GST-maspin or 70 μg GST. The follow-up treatment of tumors was carried out by subcutaneous implantation of the pellet at days 15 and 30 within 0.3 cm of the tumor site and the incision sealed after implantation. At the end of each experiment (7–8 weeks), the tumors were measured, excised and fixed in 10% neutral buffered formalin. Samples were then embedded and sectioned to 5 μm for histology (hematoxylin and eosin) and immunohistochemistry. For immunohistochemistry, monoclonal antibodies to CD 31 (Pharmingen, Sandiego, California) was used at 1:50 dilution at 4 °C overnight. After rinsing, slides were incubated with the secondary goat antibody to rat at a dilution of 1:100 for 1 h at room temperature. Slides were then rinsed and incubated with an avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories, Burlingame, California), followed by DNBA (3, 3'-Diaminobenzidine Tetrahydrochloride) color development. Vessels were counted as described [20] by first scanning the sections at the low power for hot spots or high vascular density (×40), and then counting the areas of microvessels at high power (×400). Microvessel density was calculated by adding the numbers from three hot spot fields with a ×400 objective.

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Maspin Plays an Important Role in Mammary Gland Development

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Maspin is a unique member of the serpin family, which functions as a class II tumor suppressor gene. Despite its known activity against tumor invasion and motility, little is known about maspin's functions in normal mammary gland development. In this paper, we show that maspin does not act as a tPA inhibitor in the mammary gland. However, targeted expression of maspin by the whey acidic protein gene promoter inhibits the development of lobular–alveolar structures during pregnancy and disrupts mammary gland differentiation. Apoptosis was increased in alveolar cells from transgenic mammary glands at midpregnancy. However, the rate of proliferation was increased in early lactating glands to compensate for the retarded development during pregnancy. These findings demonstrate that maspin plays an important role in mammary development and that its effect is stage dependent. © 1999 Academic Press

Key Words: **maspin; serpin; lobular–alveolar structure; mammary development; whey acidic protein.**

INTRODUCTION

Serine protease inhibitors (serpins) comprise a large family of molecules that play a variety of physiological roles *in vivo*. Serpins can be divided into two categories: inhibitory and noninhibitory serpins. Noninhibitory serpins, typified by ovalbumin and PEDF, do not exhibit protease inhibitor activity, but rather function as a storage protein and inducer of cell differentiation, respectively (Hunt and Dayhoff, 1980; Tombran-Tink *et al.*, 1992). Inhibitory serpins ablate serine proteases through their functional domain-reactive site loop (RSL) (Potempa *et al.*, 1994). Interestingly, some inhibitory serpins have evolved other regulatory functions. For example, plasminogen activator inhibitor 1 (PAI-1) not only specifically inhibits tPA and uPA, but also regulates cell adhesion, which is independent of its protease inhibitor function, by blocking integrin $\alpha v\beta 3$ binding to vitronectin (Stefansson and Lawrence, 1996; Deng *et al.*, 1996).

Maspin is a unique member of the serpin family that shares extensive homology with PAI-1 and other serpins (Zou *et al.*, 1994). Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary carcinoma cells in culture (Zou *et al.*, 1994; Sheng *et al.*, 1996; Zhang *et al.*, 1997a). Tumor transfectants expressing maspin exhibit decreased growth and metastasis in nude mice (Zou *et al.*, 1994). Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness (Zhang *et al.*, 1997b). In human breast tissue, maspin is produced predominantly by myoepithelial cells and it has been suggested that these maspin-expressing cells form a defensive barrier for the progression from ductal carcinoma *in situ* to more invasive carcinomas (Sternlicht *et al.*, 1997).

Structurally, maspin has a unique RSL different from that of other inhibitory serpins (Fitzpatrick *et al.*, 1996; Hopkins and Whisstock, 1994). It has been shown that the short RSL in maspin presents a structural difficulty that would not allow maspin to undergo the stressed-to-relaxed transition, a feature characteristic of noninhibitory serpins (Pemberton *et al.*, 1995). However, a recent report by Sheng *et al.* demonstrated that maspin could specifically function as a protease inhibitor by blocking tissue plasminogen activator *in vitro* (Sheng *et al.*, 1998). This observation led to the conclusion that maspin's anti-protease activity was responsible for its ability to inhibit tumor cell invasion and

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motility (Sheng *et al.*, 1994, 1998). However, the biological relevance of a maspin/tPA interaction has not been confirmed *in vivo*.

The murine mammary gland undergoes cycles of growth, morphogenesis, and involution throughout development. Interactions between epithelial cells and the extracellular matrix (ECM) play an essential role in mammary gland development (Talhouck *et al.*, 1991; Sympson *et al.*, 1994). Disruption of cell adhesion disrupts normal mammary morphogenesis and differentiation (Faraldo *et al.*, 1998). Indeed, destruction of the ECM by proteases, which disrupt cell–cell and cell–basement membrane contacts, has a similar defect in mammary development (Alexander *et al.*, 1996; Sympson *et al.*, 1994; Talhouk *et al.*, 1992; Witty *et al.*, 1995). To delineate maspin's function *in vivo*, we have generated transgenic mice that overexpress maspin in the mammary gland under the control of the WAP (whey acidic protein; Hennighausen *et al.*, 1982) gene promoter. Since maspin is expressed in mammary epithelial cells, as is the WAP gene, and WAP expression begins at midpregnancy and remains on throughout lactation (Pittius *et al.*, 1988), this study assessed the effect of maspin transgene expression on normal mammary gland development during this time frame. Our data demonstrate that overexpression of maspin by the WAP promoter inhibits alveolar development and differentiation.

MATERIAL AND METHODS

Mice

For phenotypic comparisons (histology, RNA, protein), all mice in this study were age matched with wild-type littermates as controls. Virgin mice were between 7 and 8 weeks. Unless otherwise indicated, mice between 7 and 8 weeks of age were used for mating. Timed pregnancy was determined by plug appearance and confirmed by delivery. Involution was forced by removing the pups from the mother at lactation day 10 except for the RT-PCR assay, when pups were taken from mother at lactation day 21. For each time point, samples from at least two transgenic and wild-type mice were examined.

Zymogram Gel

The activity of plasminogen activators (tPA and uPA) in mammary gland was detected in casein gels supplemented with plasminogen as described by Talhouk *et al.* (1991). Fresh samples from mammary tissues were frozen in liquid nitrogen. Tissue was then pulverized into a fine powder and suspended 1:5 (wt/vol) in extraction buffer (1% Triton X-100, 500 mM Tris–HCl buffer, pH 7.6, 200 mM NaCl, 10 mM $CaCl_2$). The suspension was frozen on dry ice and thawed four times and microfuged (12,000*g* for 30 min). The supernatant was used for zymogram gel. Each sample loaded contained 15 μg protein. For casein gels, 12% polyacrylmide with 1 mg/ml purified casein (Sigma, Inc., St. Louis, MO) was prepared with plasminogen (6 μg/ml; American Diagnostica, Inc., CT). Purified single-chain tPA was purchased from American Diagnostica and 0.2 ng (0.1 IU) tPA was used per lane (commercial tPA sample contains a small amount of uPA activity). Recombinant maspin was prepared as described before (Zhang *et al.*, 1997a) and concentrated by centrifugation in Centracon (Micron, Inc., MA). The tPA present in mammary gland (15 μg extract) was quantitated by comparing its activity to that of a purified tPA control. The level of tPA in virgin or pregnant mammary gland (15 μg extract) was estimated at about 0.1 ng. To characterize the effect of maspin on plasminogen activators, mammary protein extracts or purified tPA (0.2 ng) were either incubated with concentrated maspin (2 μg in 15-μl reaction solution of 2.6 μM) or mock treated for 30 min at 37°C before mixing with 4× SDS sample buffer. The molar ratio of maspin to tPA was calculated to exceed 20,000 to 1 in reactions containing mammary extracts. After electrophoresis, the zymogram gel was incubated in a sealed plastic bag with concentrated maspin at 10 μM or as a control with PMSF at the recommended concentration of 5 mM for a period of 24 h in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.6. The gel was then stained with Coomassie Blue R250 for 45 min and photographed.

Whole-Mount Preparations

For whole-mount comparison, two samples from each time point were examined. Mammary gland whole mounts were performed by spreading the fourth inguinal gland on a glass slide followed by fixation in Carnoy's solution for 60 min at room temperature (Medina, 1973; Li *et al.*, 1996). Following fixation, the gland was dehydrated by 70% EtOH, followed by staining with carmine alum overnight (0.2% carmine, 0.5% aluminum sulfate). The tissue then was dehydrated and mounted on glass slides using routine methods.

Histology and Immunostaining

Mammary glands were removed under anesthesia from normal and transgenic females at different stages of development. Unless mentioned in the text, the samples were taken from left or right inguinal No. 4 mammary gland for histology. Mammary tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. PCNA staining was performed as recommended by the manufacturer (Zymed, Inc., San Francisco, CA). TUNEL assay was carried out as described by Li *et al.* (1995) utilizing a TDT nick end-labeling kit by Boehringer Mannheim (Boehringer Mannheim, Inc., Mannheim, Germany).

Generation of Transgenic Mice

The WAP–maspin vector (Fig. 3A) was generated by PCR amplification of the mouse maspin coding region (Zhang *et al.*, 1997a) with primers flanked by *Kpn*I and *Sal*I sites (5′-CGGTACCGGATCCATGGATGCCCTGAGACTGGCA-3′ and 5′-TCCCCCGGGTCGACTACAGACAAGTTCCCTGAGA-3′). After ligating the mouse maspin cDNA between WAP exon 1 and 3, the maspin cDNA portion was sequenced to ensure the absence of mutations. The vector was digested with *Not*I and *Hin*dIII and the lineralized WAP–maspin construct was injected into C57BL/6 × BALB/c F_1 fertilized eggs. The injected embryos were transferred into oviducts of recipient pseudo-pregnant female C57BL/6 mice, and the offspring were analyzed by Southern blot. Six transgenic founders were generated and crossed with C57BL/6 mice to generate F_1 lines. Two of the best expressing lines of maspin transgenics were established for further analysis, and positive litters were screened by PCR analysis using the following primers. Endogenous maspin (199 bp): 5′ primer, GATGGTGGTGAGTCCATC; 3′ primer, TC-

CCCCGGGTCGACTACAGACAAGTTCCCTGAGA. Transgene (218 bp): 5′ primer, GATGGTGGTGAGTCCATC; 3′ primer, GCTCTAGAGGTGTACATGTCATGACACAGTCGAC.

For RT-PCR identification of transgene and endogenous maspin mRNAs (RT-PCR is not used to quantitate the difference of expression level in this case), the 3′ primers of transgene and endogenous maspin above were used for reverse transcription. Both transgene and endogenous maspin products were PCR amplified with the above sets of primers. PCR was carried out for 35 cycles in two sets of identical reaction conditions (95°C for 1 min, 52°C for 1 min, 72°C for 1 min 30 s, 34 cycles).

Northern Blot Analysis

RNA was isolated by guanidine as previously described (Swisshelm *et al.*, 1994). Total RNA was fractionated on 1% agarose–1.7 M formaldehyde gels, transferred to a Zetaprobe (Bio-Rad Laboratories, Richmond, CA) membrane in 20× SSC, and baked for 1 h at 80°C. Blots were probed with a 1.5-kb *Eco*RI/*Xho*I fragment from the mouse maspin cDNA plasmid. Ribosomal protein gene 36B4 was used as an internal loading and transfer control (Laborda, 1991).

Western Blot Analysis

The mouse mammary tissues were frozen in liquid N_2 and lysed in electrophoresis sample buffer (Zou *et al.*, 1994; Zhang *et al.*, 1997a), and the extracts were subjected to electrophoresis on a 10% SDS–polyacrylamide gel and transferred to Immobilon membrane. For β-casein and WAP protein analysis, 20 μg of whole extract for each sample was loaded for transfer. The blot was exposed first to anti-WAP antibody and reexposed to anti-β-casein antibody. For anti-WAP antibody, a 1:1000 dilution was used and followed by exposure to a 1:2500 dilution of peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Amersham, Arlington Heights, IL) as described (Li *et al.*, 1996). Anti-rat β-casein monoclonal antibody (kindly provided by Dr. Jeff Rosen's laboratory) was originally a gift from Dr. Mina Bissell. A dilution of 1:2500 was used for this antibody followed by an exposure to a 1:2500 dilution of sheep anti-mouse IgG (HRP conjugated) (Amersham). The blot was finally probed with an anti-β-actin monoclonal antibody (Sigma) at a 1:2500 dilution followed by an exposure to a 1:10,000 sheep anti-mouse IgG (HRP conjugated) (Amersham).

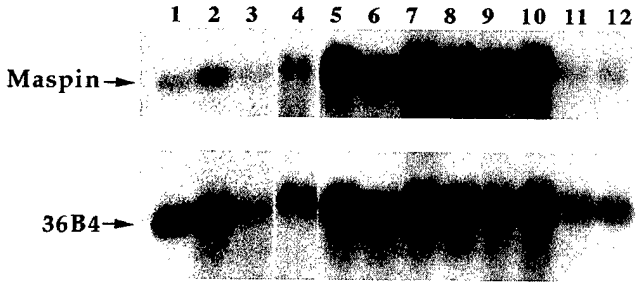


FIG. 1. Northern blot analysis of maspin gene in normal mouse mammary glands during development. Each lane contains 10 μg of total RNAs. Samples are from virgin (lanes 1, 2), day 10 pregnant (lanes 3, 4), day 19 pregnant (lanes 5, 6), day 3 lactation (lanes 7, 8), day 3 involution (lanes 9, 10), and day 10 involution (lanes 11, 12). The blot was hybridized with a cDNA probe for mouse maspin. The ribosomal protein gene 36B4 was used as a loading and transfer control.

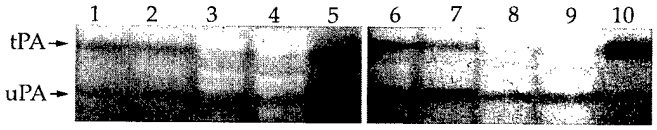


FIG. 2. The effect of maspin on plasminogen activator activity during mammary gland cycles assayed by SDS–substrate zymography. Mammary samples from 8-week-old virgin (lanes 1, 6), day 15 pregnancy (lanes 2, 7), day 3 lactation (lanes 3, 8), day 3 involution (lanes 4, 9), and pure tPA (lanes 5, 10) were assayed in casein/plasminogen gel. Lanes 6–10 were duplicate samples of lanes 1–5 loaded on the same gel. Samples in lanes 6–10 were pretreated with maspin at 2.6 μM and the gel was continuously incubated after electrophoresis with maspin at 10 μM as described under Material and Methods. All lanes were loaded with 15 μg of mammary extracts or 0.2 ng of tPA control.

RESULTS

Maspin Does Not Act as a tPA Inhibitor in Mammary Gland

To determine the temporal expression of maspin during mammary gland development, Northern blot assays were performed (Fig. 1). Maspin mRNA was expressed at relatively low levels in virgin and early pregnant mammary glands; however, maspin gene expression increased during late pregnancy and lactation. Following involution, maspin mRNA levels decreased to those observed in the prepregnant stage (lanes 11 and 12).

To delineate whether maspin regulated protease activity in the mammary gland, SDS–PAGE containing casein and plasminogen was carried out using mammary samples obtained at different stages of development. The activities of 48- (uPA) and 68-kDa (tPA) were observed in casein/plasminogen gels as previously reported (Talhouk *et al.*, 1991; Lund *et al.*, 1996) (Fig. 2). The tPA activity was high in virgin and pregnancy and low in lactation but began to elevate 3 days after forced involution. It was estimated that the mammary gland (15 μg extract from virgin or pregnant stage) contained less than 0.1 ng tPA (see Material and Methods). As shown in Fig. 2, pretreatment of mammary extracts or pure tPA with maspin at 2.6 μM (molar ratio of maspin to tPA exceeded 20,000 to 1) and continuous incubation of the zymogram gel with maspin at 10 μM did not inhibit the activity of tPA or uPA. However, recombinant maspin used was functional against cell motility at submicromolar concentration (data not shown). As a positive control, treatment of the gel by PMSF, a nonspecific serine protease inhibitor, effectively abolished tPA and uPA activity (data not shown).

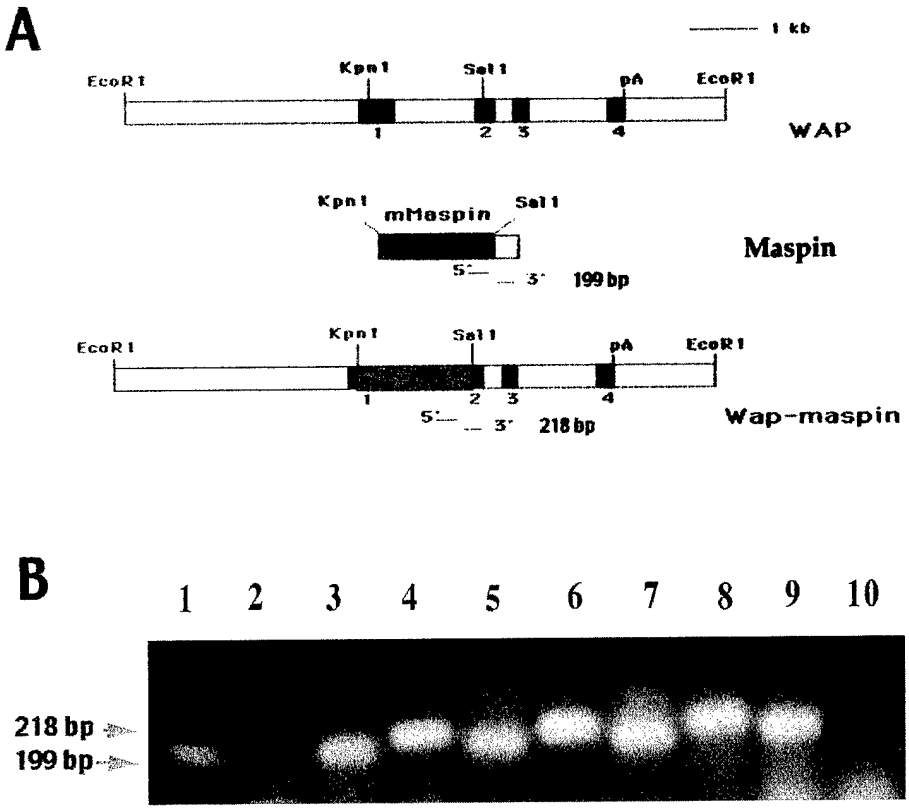


FIG. 3. WAP–maspin transgenic mice. (A) Schematic diagram of the structure of the WAP–maspin construct. WAP genomic DNA was digested by *Kpn*I and *Sal*I to remove exons 1 and 2 and intron 1. The mouse maspin (mMaspin) cDNA (gray box) was digested with *Kpn*I and *Sal*I and ligated to the digested WAP plasmid to create the WAP–maspin construct. (B) Expression of maspin in the mammary glands of transgenic mice. RNAs (2 μg) from 8-week-old virgin (1, 6), day 15 of pregnancy (2, 7), day 19 of pregnancy (3, 8), day 3 of lactation (4, 9), and day 4 of involution (5, 10) samples were analyzed by RT-PCR with two sets of primers (see Material and Methods). Samples of odd number (1, 3, 5, 7, 9) gave rise to an endogenous maspin PCR product of 199 bp, while samples of even number (2, 4, 6, 8, 10) yielded a transgene transcript of 218 bp.

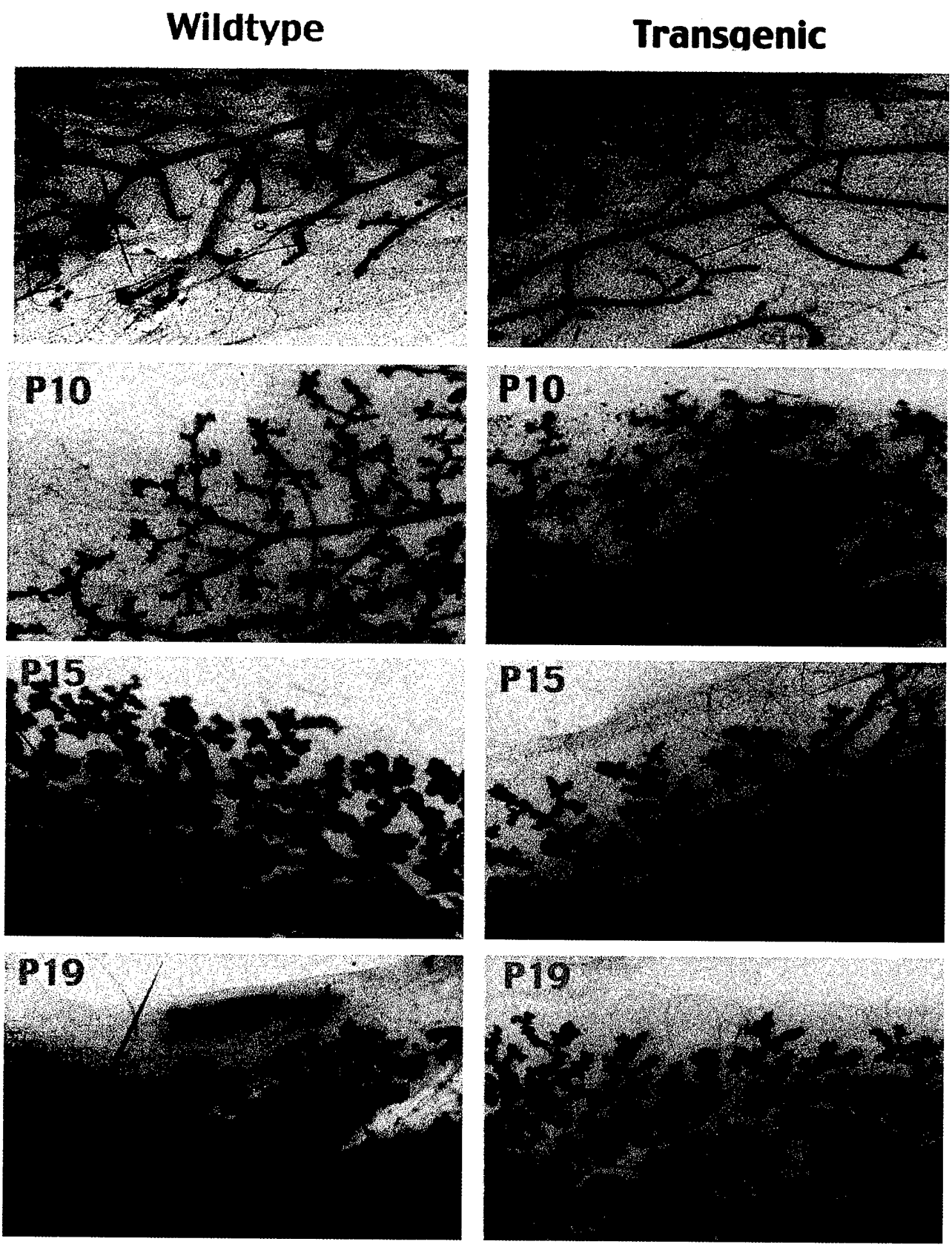
WAP–Maspin Transgenic Mice

To further investigate the role of maspin in mammary development, we utilized a transgenic mouse system to examine the effect of overexpression of maspin under control of the WAP promoter. The whey acidic protein has been shown to be exclusively expressed in mammary epithelial cells during midpregnancy and lactation.

Transgenic mice were generated by injecting embryos with the construct as shown in Fig. 3A. Screening of founders was performed by Southern blot analysis using DNA isolated from mouse tissues (data not shown). Of the six founder lines generated, two lines that displayed similar phenotypes were maintained and the results reported below were obtained from samples of the same line.

The expression of both endogenous maspin and the transgene in transgenic mouse mammary glands was examined qualitatively by reverse transcription-PCR (Fig. 3B). RNA was isolated from the mammary glands of transgenic mice at different stages of development. As shown in Fig. 3B, the PCR product of the transgene differed from that of endogenous maspin by 19 bp (218 bp vs 199 bp), allowing them to be distinguished by electrophoresis. As previously

FIG. 4. Whole-mount analysis of normal and transgenic mammary glands during development. Inguinal No. 4 glands were taken for all analyses. The virgin mammary glands (V) were from age-matched virgin transgenic and wild-type mice at 8 weeks of age. Age-matched mice were mated at 7 weeks of age for pregnancy. P10, 15, and 19 indicate days 10, 15, and 19 of pregnancy. Photographs were taken with a 4× power objective.



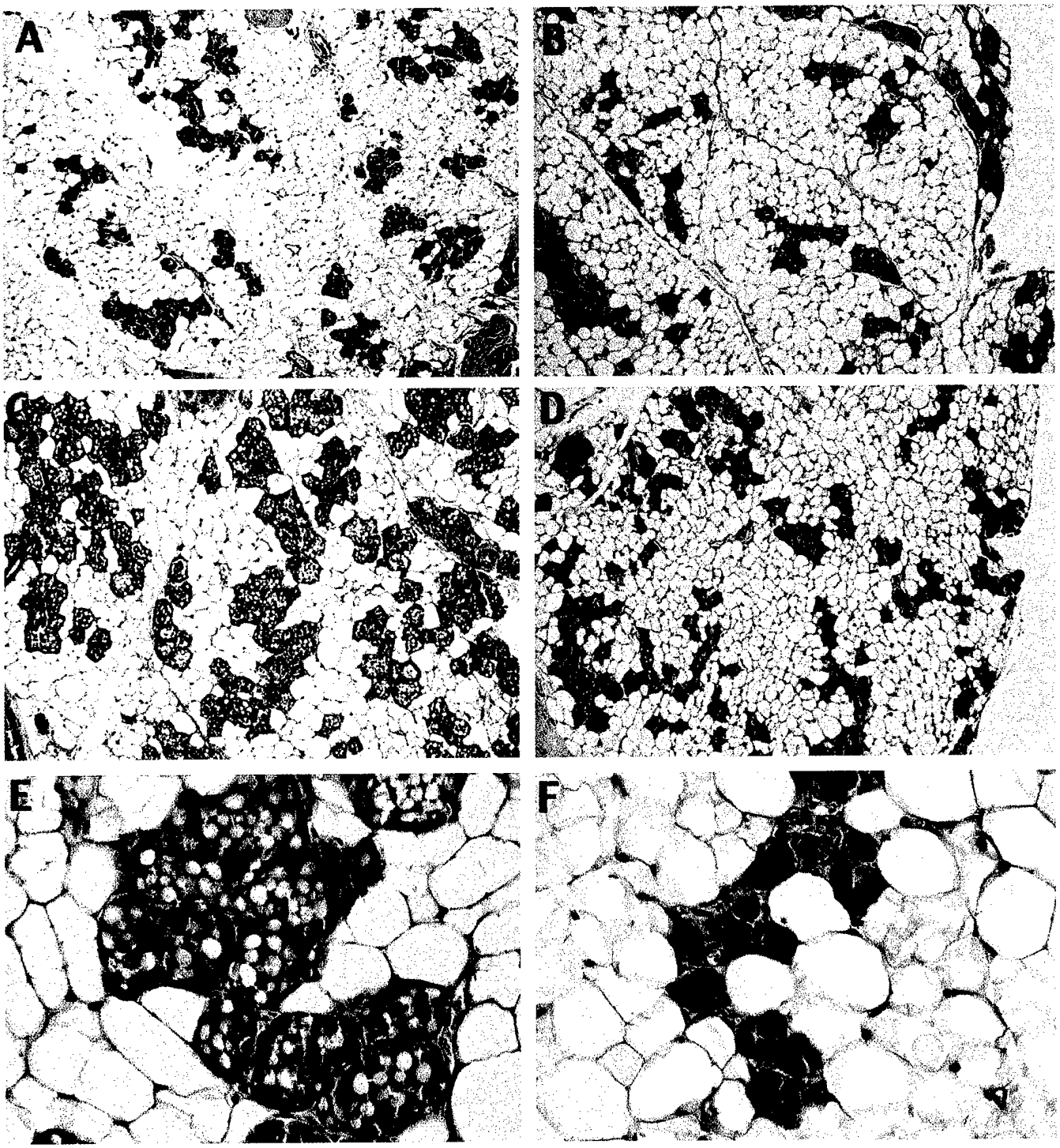


FIG. 5. Histological analysis of mammary tissues from the following mice: (A) wildtype at day 15 pregnancy, (B) transgenics at day 15 pregnancy, (C) wildtype at day 19 pregnancy, and (D) transgenics at day 19 pregnancy. (E and F) (Same magnification) High-power pictures from (C) and (D), respectively. Note the reduced numbers of alveolar structures and the smaller lumen size in the transgenics (D, F). Photographs were taken with a 10× objective for (A–D) and with a 40× objective for (E and F).

shown by Pittius *et al.* (1988), the WAP–maspin transgene was expressed during late pregnancy and lactation, but was not detectable in virgin mice and glands undergoing involution (Fig. 3B).

To characterize the mammary gland phenotype of the WAP–maspin mice, whole-mount preparations of the mammary glands from wild-type and transgenic mice were analyzed (Fig. 4). Ductal elongation and branching appeared to be normal in wild-type and transgenic virgin mice (Fig. 4). As shown in Fig. 4, no differences in alveolar structures were present between the transgenic and wild-type animals up to day 10 of pregnancy; however, minor differences did become noticeable at day 15 of pregnancy following the activation of the WAP promoter-driven transgene. These mammary glands exhibited decreased alveolar densities, which were further reduced compared to controls at day 19 and resembled the morphology of the midpregnant wild-type controls.

This defect was also observed in histological samples taken from mid- to late-pregnant transgenic mammary glands (Fig. 5). A minor reduction in the numbers of lobular–alveolar structures was noted at day 15 of pregnancy in the transgenic line (Figs. 5A and 5B). The alveolar units in wild-type glands contained large lumens that were filled with fat droplets at day 19 of pregnancy (Fig. 5C). In contrast, the mammary glands from transgenic mice contained fewer lobular–alveoli structures, and the size of each alveolar structure was greatly reduced (Figs. 5D, 5E, and 5F). In many cases, the lumens of the alveoli were closed. This defect was due to the expression of the transgene activated by the WAP promoter from midpregnancy.

Increased Apoptosis and Proliferation in Midpregnant and Early Lactating Mammary Gland of Transgenic Mice

Since the underdevelopment of the mammary glands in the WAP–maspin transgenic mice could have arisen from either decreased proliferation or increased apoptosis or a combination of the two, TUNEL and PCNA immunohistochemistry assays were carried out utilizing pregnant and early lactating mammary glands from wild-type and transgenic mice. As shown in Table 1, the apoptotic rate was significantly increased in transgenic glands at midpregnancy (2.29 ± 0.26%) compared to controls (0.91 ± 0.09%). In contrast, little difference was observed in cell proliferation at day 15 of pregnancy (Table 2). However, during lactation, the apoptosis and proliferation profiles changed significantly in the transgenic strain. Secretive alveolar cells occupied the majority of the fat pad and there was a low rate of proliferation and apoptosis in samples taken from normal mammary glands. This observation contrasted to the results obtained from the WAP–maspin mice, in which a large percentage of the fat pad was devoid of alveolar cells and an increased rate of proliferation was observed (Table 2). Although apoptosis was still higher in the transgenic glands than in normal control mice, there was a net increase in the number of alveolar cells between day 1 and day 10 lactation samples (data not shown). Both proliferation and apoptosis index decreased quickly as lactation proceeded and by lactation day 10, very few cells were PCNA-positive and apoptotic in both transgenic and normal mice.

Effect of Transgene Expression on Milk Gene Expression

The defect in alveolar structures in the WAP–maspin mice during late pregnancy severely hampered the ability of the mother to successfully nurse her entire litter. Indeed, most of the pups died due to insufficient milk production. However, these pups could be rescued by fostering them to a BALB/c nontransgenic lactating female. The number of pups that a transgenic mother could nurse varied between animals. A survey of five sibling mothers at their first pregnancy yielded an average survival rate of 3.6 pups/litter.

Since milk protein genes can function as differentiation markers for the mammary gland, we compared their expression patterns in transgenic and wild-type control mice. Western blot analysis showed that WAP and β-casein were highly expressed in wild-type mammary glands at day 19 of pregnancy and during lactation (Fig. 6). However, WAP and β-casein were not detectable in day-19 pregnant transgenic mice (lanes 8 and 1). Both milk proteins were present in lactating day 1 transgenic glands, but at a reduced levels, which increased as lactation progressed (lanes 6 and 5). This observed decrease was likely due to the effect of reduced number of alveolar cells and closed lumens in the late-pregnant transgenic mice.

DISCUSSION

We have demonstrated that maspin plays an important role in mammary gland development. Targeted expression of maspin, via the WAP promoter, inhibits alveolar development by increasing apoptosis and disrupts the process of differentiation. Since the transgene encodes the exact same maspin protein as the endogenous gene in the same cells, the phenotype is attributed directly to WAP–maspin overexpression. The phenotype clearly depends upon the level of transgene expressed, as not all of the transgenic lines exhibit the same degree of alveolar disruption, which correlates to WAP–maspin overexpression. Even though the line tested displayed the most severe phenotype, due to the patchy expression pattern of the WAP transgene (Sympson *et al.*, 1994), there are variations in alveolar structure, rate of proliferation, and apoptosis (Tables 1 and 2). This also resulted in decreased expression of endogenous maspin. In some late-pregnant mammary glands that were severely underdeveloped in the transgenic mice, overall maspin levels were down because of the drastic decrease of epithelial cells via increased apoptosis. Therefore, we did not use total maspin protein as a marker in our characterization.

TABLE 1
The Rate of Apoptosis in Wildtype and Transgenic Mice

	WT	Transgenic	*P* value
15-day pregnant	0.91 ± 0.09 (3)	2.29 ± 0.26 (3)	$P < 0.01$
19-day pregnant	0.54 ± 0.05 (2)	0.91 ± 0.54 (3)	$P < 0.04$
Day 1 lactation	0.15 ± 0.05 (2)	0.79 ± 0.11 (3)	$P < 0.01$

Note. Values are presented as percentages of apoptotic cells (means ± SD). Number of animals analyzed is indicated in parentheses. About 1000–1200 cells per sample were counted. Statistical analysis was done by the Student *t* test.

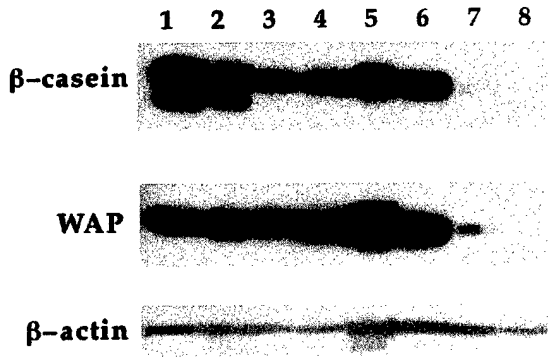


FIG. 6. Western blot analysis of milk proteins in the mammary glands of transgenic and nontransgenic C57BL/6 mice. Lanes 1–4 were from C57BL/6 mammary tissues and lanes 5–8 were from transgenic mice. Age-matched samples used were from day 19 of pregnancy (lanes 1, 8), day 1 of lactation (lanes 2, 7), day 3 of lactation (lanes 3, 6), and day 10 of lactation (lanes 4, 5). Aliquots of whole-cell extracts (20 μg) were loaded on each lane and separated by 10% SDS–PAGE and transferred to PVDF membrane. The blot was probed with antibodies against WAP, β-casein, and β-actin. β-Actin was used as a loading and transfer control.

Maspin Does Not Act as a tPA Inhibitor in Mammary Gland

Previous biochemical analysis demonstrated that maspin could interact with single-chain tissue plasminogen activator and inhibit the conversion of plasminogen to plasmin with a K_i value of 0.13 μM (Sheng *et al.*, 1998). Under similar assay conditions, PAI-1 and PAI-2 also inhibited tPA with a value either smaller than (PAI-1, 0.033 μM) or similar to (PAI-2, 0.55 μM) maspin (Sheng *et al.*, 1998). It was first suspected that maspin might act as a tPA inhibitor during mammary gland development. However, zymography assay has excluded this possibility. In theory, inhibitory serpin generally binds to its target protease in 1:1 stoichiometry, inactivating the protease by forming a complex stable to heat or SDS denaturation (Potempa *et al.*, 1994; Pemberton *et al.*, 1995). During the time of pretreatment, the exogenous maspin was in such an excess (20,000:1) that we expected that it would at least decrease the tPA activity should tPA be neutralized by maspin. However, this treatment and further incubation of the zymogram gel for 24 h with maspin at 10 μM or about 80× K_i did not have any detectable inhibition of tPA activity. We also compared the tPA activity of transgenic and wildtype mammary glands at various stages of mammary development including midpregnancy and lactation by casein zymography. No significant difference in tPA activity was observed (data not shown). Therefore, maspin's mechanism of action is unlikely to be mediated through tPA inhibition in the mammary gland. However, we cannot rule out the possibility that maspin may inhibit other unidentified proteases in mammary gland. Alternatively, maspin may possess additional functions in mammary gland. We have recently found out that maspin acts as an angiogenesis inhibitor, and this action is independent of the RSL domain of maspin (Zhang *et al.*, in review). It becomes more clear that the multifunctionality is a common feature for maspin and other serpins (Bajou *et al.*, 1998; Deng *et al.*, 1996).

Similar to studies that have been reported for PAI-1, we believe that maspin may primarily regulate cell adhesion and motility in mammary cells, possibly by regulating integrin profiles. Indeed, expression of a dominant-negative β1 integrin in the mammary gland, which disrupted the function of β1 and its associated integrins, resulted in a phenotype similar to that observed in the WAP–maspin transgenic mice (Faraldo *et al.*, 1998). Both maspin and chimeric β1 transgene expression caused underdevelopment of the mammary gland in midpregnancy and early lactation, which was accompanied by an increase in apoptosis. In early lactation, milk protein levels were also reduced. The similarity in phenotypes suggests that overexpression of maspin may act to perturb integrin regulation or other associated cell adhesion molecules. This hypothesis is partially supported by an *in vitro* study which showed that exogenous maspin modified cell attachment to fibronection by regulating integrin profiles, including down-regulation of β1 integrin (Seftor *et al.*, 1998). However, there are some differences in the observed phenotype between these two transgenic mice strains. For example, the proliferation of alveolar cells in the β1-transgenic mice decreases during pregnancy, while maspin transgenic mice

TABLE 2
The Rate of Proliferation in Wildtype and Transgenic Mice

	WT	Transgenic	*P* value
15-day pregnant	11.73 ± 1.93 (4)	10.50 ± 2.59 (4)	$P < 0.89$
Day 1 lactation	2.73 ± 1.72 (3)	9.49 ± 1.86 (3)	$P < 0.01$

Note. Values are presented as percentages of PCNA-positive nuclei (means ± SD). Number of animals analyzed is indicated in parentheses. In all cases, 1000–1200 cells per sample were counted. Statistical analysis was done by the Student *t* test.

showed no change. Since alterations in integrin profiles, especially the dimerization of subunits, are not well characterized in the mammary gland, it is not known whether other integrins or cell adhesion molecules are regulated by maspin.

Disruption of the Process of Differentiation at Midpregnancy

During pregnancy, alveolar cells proliferate and differentiate. By parturition, the mammary gland terminally differentiates and functions mainly to secrete milk for nursing the progeny. Milk protein genes, such as WAP and β-casein, are hallmarks of this process. In the WAP–maspin transgenic mice, we observed that WAP and β-casein gene expression was diminished from late pregnancy to early lactation, demonstrating that the differentiation process is disrupted. This is reflected by the fact that the lumen of the transgenic gland is reduced in size and closed during late pregnancy compared to wild-type controls. This phenotype correlates with other *in vivo* and *in vitro* studies, suggesting that the appropriate cell adhesion interactions between the extracellular matrix and epithelial cells are required for milk gene expressions (Alexander *et al.*, 1996; Streuli *et al.*, 1995; Talhouk *et al.*, 1992).

Increased Apoptosis in Pregnancy and Proliferation in Lactation in Transgenic Mammary Gland

We have shown that WAP–maspin transgene expression significantly increases the rate of apoptosis in midpregnancy, while the proliferation rate was largely unchanged. The mechanism by which maspin regulates apoptosis is not known. One possibility is that overexpression of maspin perturbs the adhesion of alveolar cells to the ECM as does the chimeric β1 transgene and thus inhibits the motility of alveolar cells at a stage when invasion into the fat pad is critical. The proliferating alveolar cells are unable to migrate out, leading to increased apoptosis and a resulting small lumen. This is consistent with the concept that proper interactions of mammary epithelial cells with the basement membrane are essential for cell survival, and their disruption will trigger signals leading to apoptosis (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997).

During early lactation, the apoptotic rate in the WAP–maspin mice is relatively higher than in wildtype, but is lower than at midpregnancy. Proliferation is maintained at a high level, resulting in a net increase of alveolar cells. Another finding is that the lumen, which is closed in late pregnancy, opens following parturition. The lumen is likely opened in response to suckling (Li *et al.*, 1997). However, it is not clear why there is such an increase of proliferation in early lactation. In the normal gland, alveoli encompass the majority of the mammary fat pad by late pregnancy and are under the influence of estrogen and progesterone (Vonderhaar *et al.*, 1984). There is a transient surge of alveolar proliferation during early lactation, which allows these alveoli to engorge the entire gland (Vonderhaar *et al.*, 1988). In the maspin transgenic mice, a compensatory proliferation of epithelial cells was observed to compensate for the incomplete alveolar development during pregnancy in order to fulfill the physiological requirement to nurse the young.

In summary, we have demonstrated that maspin does not act to inhibit tPA, but may act primarily as a cell adhesion and motility regulator in mammary epithelial cells. To confirm this hypothesis, we are currently generating mice null for the maspin gene and assaying its role in normal mammary gland development. By delineating maspin's mechanism of action *in vivo* the role of maspin as a tumor suppressor can be elucidated.

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